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Determination and comparison rate of expression markers of osteoblast derived of Adipose derived stem cells markers in monolayer and pellet culture models

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Abstract: Nowadays high accident rates, fractures leading to permanent bone disorders and the impossibility of bone transplant have made scientists to look for new methods of repairing injured bones. Considering the application of stem cells in bone tissue engineering, there exists the necessity to investigate various culture methods and suitable fields and scaffolds. Thus, we decided to induce adipose-derived stem cells into osteoblast cells in two systems of pellet culture and monolayer and compare osteogenic markers.
Methods: Stem cells have been separated via mechanical and enzymatic methods and cultured in monolayer and pellet culture models with osteogenic medium. Then, RNA was separated from differentiated cells, complementary DNA (cDNA) was synthesized and amplified. Polymerase chain reaction (PCR) product was transferred to electrophoresis gel. The intensity of the bands was measured by Image-J software and analyzed by SPSS.
Results: average osteopontin, osteocalcin and Runx2 genes in differentiated cells in the two culture systems showed a significant difference. The expression of osteocalcin, osteopontin and Runx2 gense in pellet system were more than monolayer systems in 21 days.
Conclusion: This study indicated that pellet and monolayer culture systems are appropriate for bone engineering but osteocalcin, osteopontin and Runx2 genes expressions were different in the two culture system.

Keywords:- Adipose-derived stem cells, Osteocalcin, Runx2, Osteopontin, Pellet culture, Monolayer

I. INTRODUCTION

New approaches to clinical problems based on translational medicine start with basic research and progress 'hand-in-hand' with clinical observations. Scientists are increasingly aware that this 'bench-to-bedside' approach to translational research is really a two-way street which can strengthen and accelerate critical points of the research process. Bone defects due to trauma and to pathological and physiological bone resorption represent a major challenge and are a global health problem. The need for bone regeneration in cranial, oral and maxilla -facial and orthopedic surgery is one of the central clinical issues in regenerative and rehabilitation medicine. It is difficult to convey the enormous social and psychological handicap of persons with bone defects and the significant reduction in their quality of life. In addition to trauma, bone healing problems may be related to age, sex and infection as exemplified by diagnoses such as osteoporosis, osteopenia and severe dental problems related to loss of teeth(1).

Tissue engineering has emerged as an interdisciplinary field with tremendous potential to develop and use new knowledge based materials and cells that can be used in the fast-growing market of bone repair(1).

The potential of in vitro osteoblasts to maintain their phenotype,and their level of activity,can vary dramatically and is undoubtedly dependent on the cell type used and substrate characteristics(2).

ADSCs hold great promise for use in tissue engineering and cell based therapies. Thay have shown the capacity for multilineage differentiation to osteogenic, chondrogenic, myogenic, neurogenic, adipogenic, hepatic, cardiac and endothelial phenotypes and are more readily available than embryonic stem cells. Additionallly,ADSCs are obtained wity less morbidity and in greater numbers than bone marrow-derived stem cells.There is extensive evidence confirming the capability of ADSCs to develop into and osteogenic lineage(3-10).

The pellet culture model is commonly applied to enhance in vitro chondrogenesis of primary chondrocytes or bone marrow derived progenitor cells.For chondrocyte differentiation, the pellet culture model simulates the early condensation of mesenchymal stem cells during embryogenesis prior to the onset of chondrogenesis and the production of the extracellular matrix(ECM) by chondrocytes. Therefore, differentiation of the round-shaped cells inside the pellet is increased and apposition of ECM is significantly induced (11).

Monolayer culture is type of culture model that cells growth in single layer on flask or petri dish,this type culture is necessary for independence cells.Monolayer culture of osteoblasts is the most frequently performed method to investigate the effects of growth factors or hormones on the behavior of osteoblast-lineage cells in vitro.Lian and Stein described the processes of in vitro differentiation of at low-density seeded monolayer-cultured rat calvaria-derived osteoblasts in detail**(**12).

The studies reported that cultured hepatocyted in 3D pellet culture showed better function, differentiation and seceration of ECM compared to monolayer(14).

To evaluate the effect of ECM structure and composition on osteogenic differentiation, we compare the expression of osteogenic markers in ADSc grown under 2 different cell culture condition.ADSc were grown in osteogenic media by traditional cell culture technique(2-D cell monolayer on a plastic cell culture dish) and pellet culture system.Quantification of osteogenic marker expression, including:Osteocalcin,Osteopontin and RunX2, was obtained by real-time polymeras chain reaction(RT-PCR).

II. MATERIALS AND METHODS

Isolation of stem cells:

ADSCs were obtained from human lipoaspirates from 4 patients in alzahra hospital of Isfahan of iran and cultured as described in a previous study by Zuk et al(3).Briefly, lipoaspirates were washed extensively with sterile phosphate buffered salin(PBS) to remove contaminating debris and red blood cells. Washed aspirates were treate with 0.075% collagenase(type1;Sigma-Aldrich,St.Louis,MO) in PBS for 30 minutes at 37^0 C with gentle agitation.The collagenase was inactivated with an equal volume of control medium containing Dulbecco modified Eagle medium(DMEM; Sigma, St. Louis, MO),10% fetal bovine serum (FBS; Summit Biotechnology,Ft.Collins,CO),penicillin(100U/ml:Invitrogen,Gran,Island,NY),and stereptomycin(100 microgeram/ml:Invitrogen),and the infranatant centrifuged for 10 minutes at low speed.The cellular pellet was resuspended in control medium and filtered through a 100-micrometer mesh filter to remove debris.The filtrate was centrifuged as detailed above and plated onto conventional tissue culture paltes in control medium.Confluent cell cultures were split using 0.25% trypsin-EDTA(Mediatech,Herndon,VA).The cells were harvested between passages 2.

Preparation of Cell Culture: 2D(monolayer culture) and 3D(pellet culture) Systems For group 2D,cells were grown on 100-mm polystyrence cell-culture dishes(Sigma) in osteogenic media.Osteogenic media consist of control medium supplemented with 0.1 micromole dexamethasone,50 millimole ascorbate-2-phosphate,and 10 millimole B-glycerol phosphate(Sigma).The same medium was used in our 3D system.The resulting cell culture dishes contained $25x10^4$ cells.

RT-PCR

Osteopontin, osteocalcin and Runx2 expression was quantified in each digested sample at each time point. Total RNA was extracted from the cells using RNAx -plus solution (CinnaGen Inc Iran). First strand complementary DNA (cDNA) was prepared from 1µg the total RNA using oligo(dt)18 as a primer and Revert Aid TM First-strand cDNA synthesis kit (Fermentase) according to the manufacturer instructions.PCR reaction mixture contained: 2.5µl cDNA, 1X PCR buffer (AMS), 200µM dNTPs, 0.5µM of each primer, and 1 unit Taq DNA Polymerase (Fermentas). Amplification conditions for the PCR were: initial denaturation at 95^oC for 5 min, followed by 35 cycles of 93^oC for 30 sec, 65^oC for 45 sec and 72^oC for 40sec. The PCR products were then analyzed on 1% agarose gel and visualized by ethidium bromide staining and UV trans illuminator.

RT-PCR was performed with conditions and primers as described previously(3).Briefly, human GAPDH primers and probe(5' JOE and 3' TAMRA were purchased from Applied Biosystems(Foster City,CA).Total cellular RNA was prepared form and aliquot of the digested cell suspension using the Qiagen RNEasy Minikit (Qiagen Inc,Valecia,CA).RNA samples were treated with DNAse I(Qiagen) to digest residual genomic DNA,cDNA was prepared from each sample using the TagMan Gold RT-PCR kit for RT-PCT(Applied

Biosystems).Quatitative RT-PCT was performed using this kit according to the manufacturer and an ABI7700 Prism Sequence Detection system.Primer and probe sequences were designed by the UCLA Sequencing Cor Facility and synthesized by Biosource(Camarillo,CA).All probes were designed with a 5' fluorogenic prop6FAMand a 3' quencher TAMRA.The expression of GAPDH was used to normalize gene expression levels.

Statistical Analysis

For each time point evaluated in each of the study arms,the 3 values obtained for each group were averaged and plotted.The obtained results surveyed image J software and SPSS(version 17), An unpaired independent sample t test(GB-Stat,version 6.3) was performed to determine statistical significance between values.Statistical significance was considered for $p < 0.05$.

III. RESULTS

Cell culture

In low-density-seeded monolayer culture osteoblasts cells are known to display a flat and fibroblast-like morphology similar to ADSc in monolayer culture (Fig 1) .During activation and differentiation of pre-osteoblasts to mature osteoblasts,early osteocytes and terminally embedded osteocytes,cell morphological changes are apparent(Fig2).Active cuboidal osteoblasts can become embedded in ECM and can be transformed into star-shaped osteocytes(Fig 3). cell shape of 3D cultured osteoblasts differes from 2D cultured cells.while cells in low-density monolayer are characterized by a fibroblast-like morphology(Fig1, 2). the culture in a dense monolayer increases the chance of detecting cuboidal,irregularly-shaped osteoblasts with the presence of more than 2 cell processes connecting to neighboring cells(Fig3)

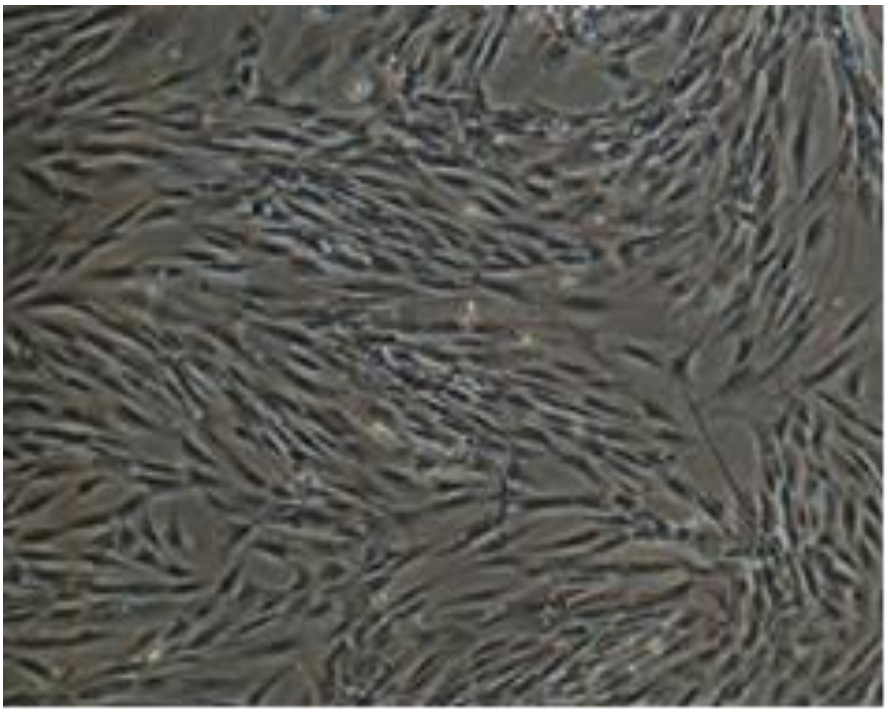

Fig 1 : Photomicrograf of alive ADSc in monolayer culture used by invert Microscop: Cells were cells with morphology similar fibroblast ×40

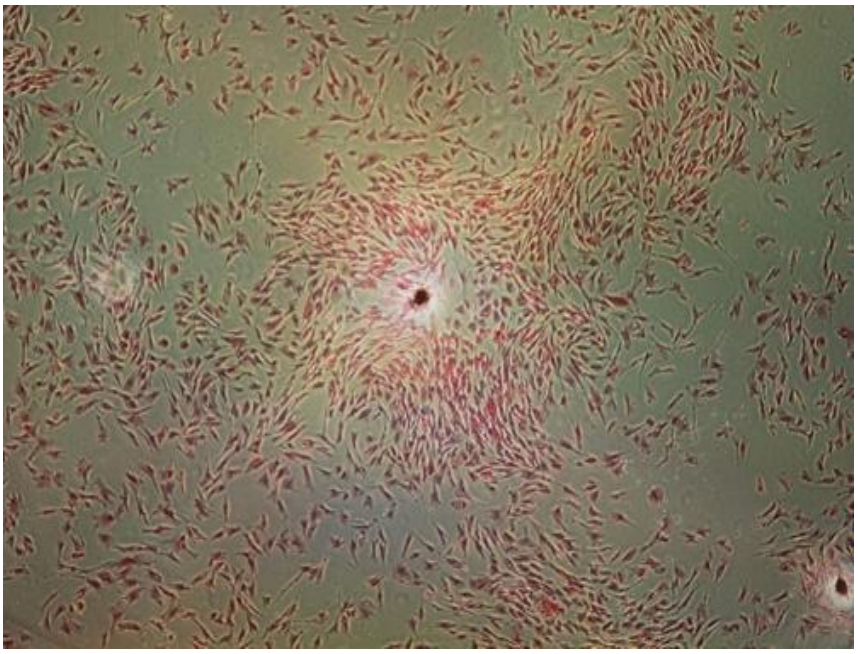

Fig2: photomicrograf of alive osteoblast cells in low density culture (day2)by invert microscop: Cells were fibroblast-like morphology in monolayer culture.. ×40

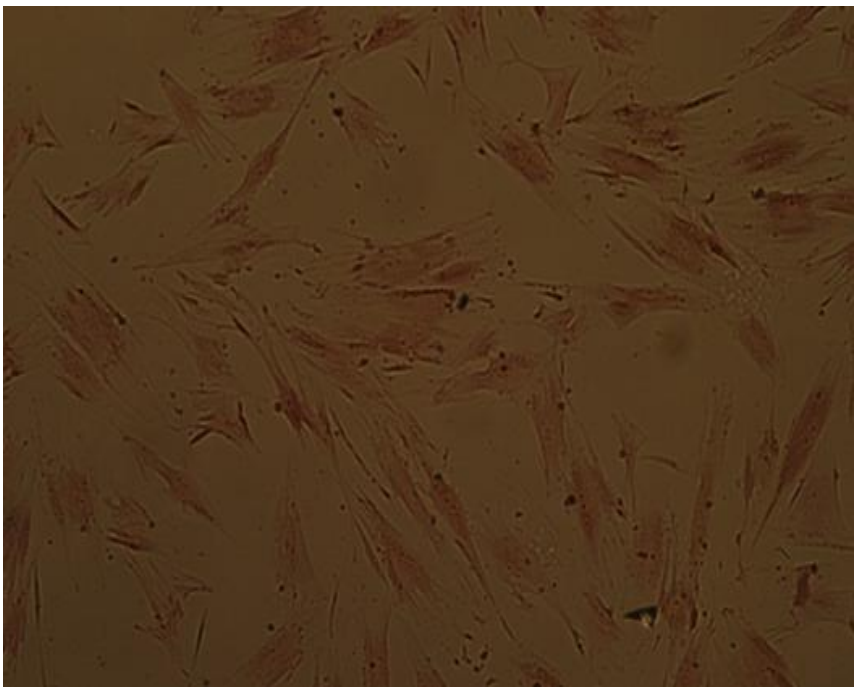

Fig3: photomicrograf of alive osteoblast cells by invert microscop: Cells was cuboid morphology in monolayer culture. Vonkossa staining. ×40

RT-PCR

At day 21,expression of osteogenic markers was quantified.In all cases osteopontin and Runx2 was greater expression by cells in 3D culture(pellet culture) than cells in 2D culture(monolayer culture)($P<0.05$) however,osteocalcin expression in pellet culture was non-significantly different to monolayer culture on day21($P<0/01$)

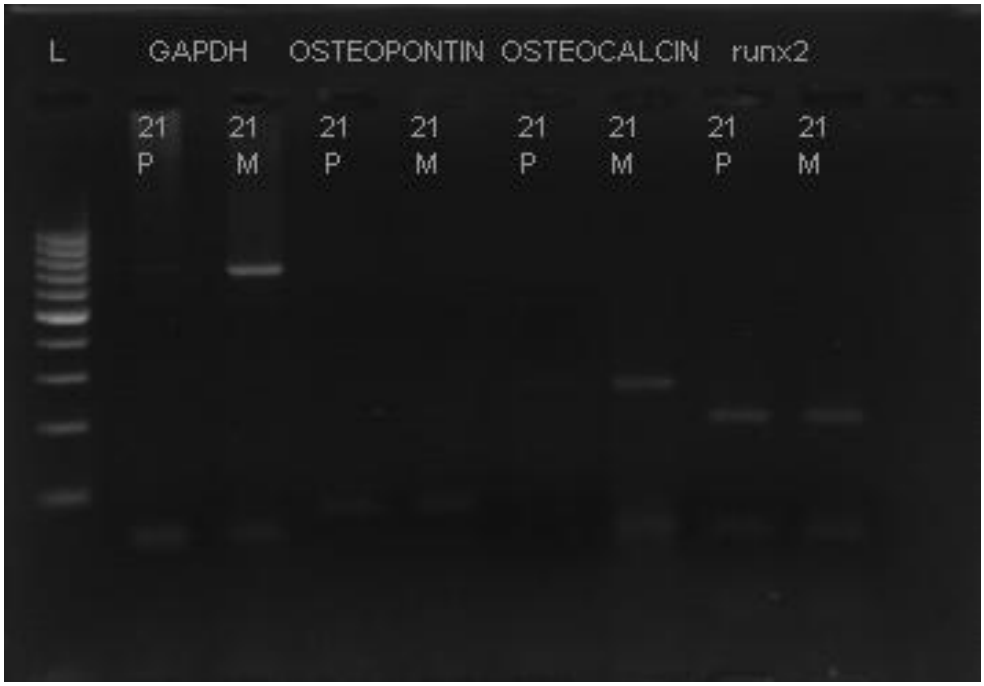

Fig 2: RT-PCR analysis of the expression of specific markers following osteogenic differentiation of ADSc. Following the osteogenic differentiation protocol, cells became positive for the expression of specific markers as osteonectin , osteocalcin and Runx , which were undetectable in control cells (GAPDH).L: 100 bp ladder.

IV. DISCUSSION

In our study rate of osteocacin,osteopontin and Runx2 genes in osteoblasts derived of ADSc in monolayer and pellet culture surveyed and comparisoned.

RT-PCR results showed that At day 21,expression of osteogenic markers was quantified.In all cases osteopontin,osteopontin and Runx2 was greater expression by cells in 3D culture(pellet culture) than cells in monolayer culture on day21.

Osteopontin (OPN) is a non-collagenous multifunctional glycoprotein routinely present in mineralized tissues (McKee and Nanci 1996), which is believed to play an integral role in cellular responses to mechanical stimuli. One recent study in OPN knockout mice revealed that OPN is necessary for the increase in osteoclastic bone resorption and decrease in osteoblastic bone formation normally associated with skeletal unloading (15-18).osteocalcin is a small calcium-binding protein found mainly in the ECM of bone, but a small amoun enters the blood(19,20).

Even though its function is still not completely understood,it is expressed by fully differentiated osteoblasts.The role of Runx2 during osteoblast differentiation and maturation is of crucial importance.Runx2 was the first transcription factor, identified through its binding site(OSE2) within the osteocalcin promoter.Its expression is up regulated during mesenchymal condensation leading to the formation of pre-osteoblasts.Therefore,Runx2 has been described as an early osteoblast differentiation marker(21).

Osteoblast differentiation goes through 3 phases: Proliferation,matrix maturation and matrix mineralizaton.Briefl,at the onset of in vitro differentiation ,spindle-sahped osteoblasts cultured in lowe-density monolayer proliferate to form a dense multilayer culture .Druing this stage, the cells undergo morphological changes.The start of the second phase-matrix maturation is characterized by an up-regulation of alkaline phosphatase activity.Reaching a constant cell number, characterized by a balance between cell proliferation and cell death,osteoblasts start to produce non-collagenous extracellular matrix proteins,such as osteopontin and osteocalcin.The maturation of the synthesized ECM is finalized with the incorporation of hydroxyapatite crystals within the matrix.The progression of osteoblast maturity is further complicated by the potential of the cells to become embedded with the matrix and further differentiate into osteocytes(12).

The pellet culture model used for increase chondrogenesis of primary chondrocyte or bone marrow stem cell currently.this type culture model,induced mesenchymal stem cells and increase secration of ECM(23).

Frunkle and et al,were cultured osteoblasts cells on alginate scaffold and compared with monolayer culture.The results demonstrated that proliferation and expression of osteocalcin and osteopontin genes on alginate scaffold is higher compared to monolayer culture model. Researchers suggested osteoblasts required regular passage for more proliferation and secretion of ECM(23).In our study,expression of the osteogenic

factores osteocalin,osteopontin and Runx2 was up-regulated in pellet culture model compared to monolayer culture model on day 21.

We predicted that an increase in secretion of ECM ,as seen during osteoblast pellet culture,would result in immensely increased osteoblast differenction.

Jahn and et al,cultured osteoblaste isolated of human hip on pellet and monolayers culture models.they reported that expression of osteocalcin and 1,2 types collagenases in pellet culture was significantly higher compared to monolayer culture.this study was like our results.thay suggested that in first osteobalstes proliferated in pellet culture then maturated because osteoblasts maturated and committed into secretion of ECM(24).

Rat calvaria-derived osteobalstes cultured in pellet culture decreased proliferation osteoblasts and started seceration ECM.The results of Owen et al suggested a functional relationship between the inhibition of proliferation and the induction of genes associated with cell differentiation and matrix maturation(25).

Bellows et al showed that formation of nodules ,which simulate a micromass,during osteoblast culture results in an increase in osteoblast maturation(26).

Shin-Yeu Ong and et al differentiated MSc into hepatocyte and cultured into monolayer and pellet culture medels .they reported that hepatocytes in pellet culture secreted albumin and urea,stored glycogen higher compared to monolayer culture. suggested that 3D pellet culture allows stable cell anchorage,permits the retention of ECM molecules produced by the cells,and as high-density cultures can be readily implanted into the liver or used in bioartificial liver devices(13).

According to our hypothesizes and else studies, maybe because of first, closer contact of cells and their secretions matrix .Second, prolongated of differentiation priod and seceration of ECM in monolayer culture.Third, a functional relationship between the inhibition of proliferation and the induction of genes associated with cell differentiation and matrix maturation.

V. CONCLUSION

We demonstrated that pellet-cultured human osteoblasts differentiated from ADSCs up-regulated osteopontin,osteocalcin and Runx2 compared to monolayer culture on day21.We believe the pellet culture model for human primary osteoblasts offers the potential further studies aiming to use ADSCs and pellet culuter model for regeneration of bone defects of in vivo.

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